Frequency and Enumeration of *Campylobacter* Species from Processed Broiler Carcasses by Weep and Rinse Samples

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ABSTRACT Frequency and numbers of *Campylobacter* spp. were assessed per freshly processed, contaminated broiler carcass. *Campylobacter*-positive flocks were identified by cecal sample analysis at slaughter. These flocks had been tested as *Campylobacter* negative at 4.1 ± 0.9 d prior to slaughter. Levels of contamination were estimated using 2 sampling approaches per carcass: (1) free weep fluids and (2) whole-carcass, 100 mL of distilled water rinses. Estimations of counts were determined by directly plating dilutions of weeps and rinses onto Campy-Cefex agar and incubating the plates at 41.5°C under microaerobic atmosphere. Confirmation was provided by latex agglutination to quantify levels per milliliter of weep and per 100 mL of rinse. Thirty-two slaughter groups (~20 carcasses per group) were compared from 2003 to 2004. The *Campylobacter*-positive weep frequency was 84.8%, whereas the frequency for rinse samples was 74.4% ($P < 0.001$). Enumeration of *Campylobacter* spp. on positive samples ranged from 0.70 to 6.13 $\log_{10}$ cfu/mL of weep (geometric mean of 2.84) and from 2.30 to 7.72 $\log_{10}$ cfu/100 mL of rinse (geometric mean of 4.38). The correlations between weep and rinse were 0.814 with 0.5 mL of rinse and 0.6294 when applying 0.1 mL of rinse The quantitative regression analyses for these 2 corresponding tests were $\log_{10}$ rinse (for 0.5 mL of inoculum) = 1.1965 $\log_{10}$ weep + 0.4979, and $\log_{10}$ rinse (for 0.1 mL of inoculum) = 1.322 $\log_{10}$ weep – 0.1521. *FlaA* SVR sequencing of isolates indicated that the same genotypes were found in weep and rinse samples. Weep and rinse sampling led to different proportions of *Campylobacter*-positive carcasses detection, but we demonstrated that this difference was reduced by increasing the amount of rinse fluid used for plating.

Key words: *Campylobacter*, frequency, enumeration, weep, rinse

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INTRODUCTION

The epidemic outbreak of human campylobacteriosis in Iceland during the late 1990s suggested that novel sources of *Campylobacter jejuni* had been introduced, causing increased public exposure (Stern et al., 2002). Among the earliest suspects fresh, chilled poultry products appeared to be plausible. In part, this was due to increased consumer demand for such products. Before February 1996, retail stores in Iceland had exclusively sold frozen carcasses to consumers, and no fresh poultry had been available in Iceland. The upsurge in consumer demand for fresh broiler products peaked in 1999, as did the incidence of human campylobacteriosis.

One consequence of selling only frozen carcasses was the reduced exposure to greater numbers of the organism via broilers. Freezing of broiler carcasses resulted in an approximate 2log reduction of *C. jejuni* numbers (Stern et al., 1985). Among 5 lots of processed broilers, levels of naturally contaminated *Campylobacter* on carcasses were reduced by log mean values ranging from 0.65 to 2.87 after 31 d of storage under frozen conditions (Georgsson et al., 2006). In 1995, regulations changed in Iceland, along with increased consumer demand for fresh product, and, consequently, the public was exposed to greater amounts of fresh poultry having increased levels of the pathogen.

Therefore, we wished to determine the frequency and numbers of *Campylobacter* associated with freshly processed broilers, which represents a measure of consumer risk. Although the regulatory measure used to monitor microbiological quality of broiler carcasses has traditionally been rinse sampling, discussions and presentations

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at various meetings involving the Food and Drug Administration, Centers for Disease Control, and Health Canada have suggested that poultry weep sampling might serve as a superior measure to assess public exposure to *Campylobacter* associated with product (R. Lowman, Canadian Food Inspection Agency, personal communications). Musgrove et al. (2003) compared weep and rinse procedures for qualitative detection of the organism and found no significant difference between the methods. The present paper addresses how weep and rinse from poultry carcasses compare qualitatively and quantitatively to one another.

MATERIALS AND METHODS

Sampling

In Iceland, regulations require that broiler flocks are sampled for *Campylobacter* spp. colonization 4.1 ± 0.9 d before slaughter. Those flocks that were determined as colonized were processed at the end of the work week (or day), and such lots were frozen or further processed to reduce subsequent consumer exposure to the pathogen. Some of the flocks that had tested as *Campylobacter* negative subsequently became colonized before the day of processing. These market-age flocks (36.4 ± 2.5 d of age) were detected by culturing 4 pools of 10 bird ceca obtained at the processing plant. Because it was not known whether the ceca would be positive, 18 to 20 freshly processed carcasses (2 cartons of 9 or 10 whole carcasses each) per lot were stored at –2 to 2°C for <24 h until the test results were determined. When the ceca were positive, the chilled carcasses were then shipped to the laboratory and tested as follows. In total, 32 process lots were colonized and sampled during the course of the study.

Immediately after the lot was processed, carcasses were placed into ready-to-ship commercial containers and held in storage. At up to 30 h after processing, the accumulated free fluids were aspirated by a sterile pipet and surface plated directly. Subsequently, each carcass was aseptically transferred into a new sterile plastic bag, 100 mL of distilled water was added, and each carcass vigorously shaken by hand for ~60 s. After being rinsed, the carcass was aseptically removed, and the rinse suspension was used for subsequent microbiological analysis.

Microbiology

Serial, logarithmic dilutions were made using sodium peptone water for the weep samples and distilled water for the rinse samples and then plated onto Campy-Cefex agar (Stern et al., 1992). Each sample was quantitatively analyzed for *Campylobacter*. Volumes of 0.1 or 0.5 mL (rinse samples) from the undiluted samples and 0.1 mL of 10^{-1} to 10^{-4} dilutions were surface plated onto duplicate Campy-Cefex plates. Before inoculation, plates to receive 0.5 mL were dried for 40 min at 37°C to obtain separated colonies. Other plates were dried for 20 min at 37°C. The inoculated plates were held in zip-sealed plastic bags and incubated at 41.5°C for 48 h. A microaerobic condition was created using gas mixtures of 5 to 10% O_2, 70 to 85% N_2, and 10 to 20% CO_2 expelled through an inserted hose to thoroughly purge the bags of the normal atmosphere. After incubation, suspect colonies, preferentially from plates containing 15 to 150 colonies, were counted, and 3 colonies from each plate were confirmed using colony morphology, Gram stain, biochemical tests, and latex agglutination assays. The results were calculated as colony-forming units of *Campylobacter* spp. per 100 mL of distilled water rinse fluid or per milliliter of the weep fluid.

Molecular Typing

Isolates collected from broiler ceca, weep sampling, and rinse sampling of 2 broiler flocks were typed using the *flaA* short variable region (*flaA* SVR; Meinersmann et al., 1997). DNA was prepared from frozen stocks by placing 10 μL of glycerol stock in 50 μL of sterile, distilled water. Cells were lysed at 100°C for 5 min. Microcentrifuge tubes containing DNA samples were spun briefly to precipitate cellular debris (Hiett et al., 2002). The DNA was prepared for unrecoverable isolates by pipetting 40 μL of Wang's transport medium into 200 μL of sterile distilled water, boiling these samples at 100°C for 5 min, and spinning to precipitate cellular debris (Callicott et al., 2005). In both cases, the *flaA* SVR was then amplified using the primers Fla4F (5′-GGA TTT CGT ATT AAC ACA AAT GGT GC-3′; Nachamkin et al., 1993) and FlaA625RU (Meinersmann et al., 1997) with a reaction mix containing 2 m*M* $MgCl_2$, 0.125 μ*M* of each primer, 0.8 m*M* each of dNTP, and 2.5 U of AmpliTaq in a 100-μL reaction. Tubes were subjected to 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by a 5-min extension at 72°C. Sequencing was performed using degenerate primers Fla106F (5′-GAY GAT GCT TCW GGK ATG-3′) and FlaA625RU by BigDye@ terminator 3.1 chemistry (Applied Biosystems, Foster City, CA). Sequence data were obtained using a 3730 DNA Analyzer (Applied Biosystems). The 359-nucleotide region between primers FlaA242FU (Meinersmann et al., 1997) and FlaA625RU was used for allelic comparisons.

Statistical Analysis

Statistical analysis of the levels of *Campylobacter* in the weep vs. the rinse sample was assessed (Microsoft Excel, Microsoft Corp., Seattle, WA). McNemar's test (95% confidence interval) was used to determine whether the proportions of positive tests were different from one sampling method to the other. Regression analyses for weep vs. rinse sample enumeration were determined for 0.1- and 0.5-mL rinse aliquots inoculated per plate.

RESULTS

In total, 615 carcasses from broilers of flocks, tested for colonization by *Campylobacter* spp., were compared

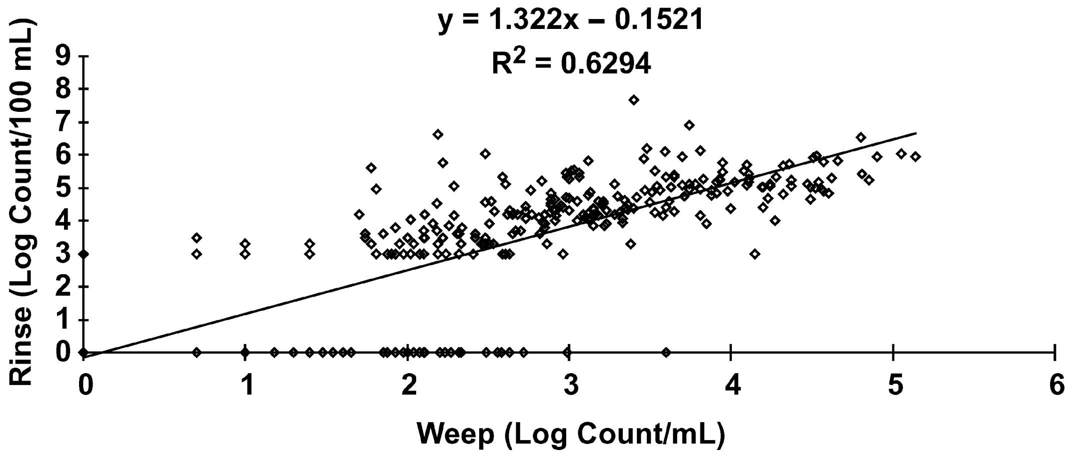

Figure 1. Regression analysis of rinse and weep log count relations from 330 broiler carcasses contaminated with *Campylobacter* spp. as measured per milliliter of weep fluids or by enumerating 0.1 of 100 mL of rinse fluids inoculated onto Campy-Cefex agar.

quantitatively and qualitatively for contamination. Figures 1 and 2 provide quantitative comparisons of weep vs. rinse sampling. The regression coefficient of 0.6294 indicated that there was good comparability between these 2 quantitative measurements when only 0.1 mL of rinse was plated from the undiluted rinse. The next year we monitored the weep samples as above, but determined the rinse samples by directly plating 0.5 mL of the undiluted rinse onto Campy-Cefex plates. The regression coefficient of 0.814 indicated that there was substantially improved quantitative comparability between these 2 measurements. The quantitative regression analyses for these 2 corresponding tests were $\log_{10}$ rinse (for 0.1 mL of inoculum) = 1.322 $\log_{10}$ weep + 0.1521 and, $\log_{10}$ rinse (for 0.5 mL of inoculum) = 1.1965 $\log_{10}$ weep + 0.4979.

The McNemar test was used to compare proportions of positive samples in the paired studies. The McNemar test indicated that qualitative weep sampling detected more *Campylobacter*-positive carcasses than did the rinse sampling. When weep sampling was compared with 0.1 mL of rinse sampling, the weep sampling detected 18.5% more *Campylobacter*-positive carcasses with a 95% confidence interval (Table 1) on the difference of 14.1 to 22.8%. When weep sampling was compared with 0.5 mL of rinse sampling, the weep sampling detected 3.7% more *Campylobacter*-positive with a 95% confidence interval of 0.7 to 6.8%. The gap reduction in differences when using more rinse fluid was correlated by the McNemar test. Results indicated the data went from χ^2 = 57.3 when comparing weep with rinse with 0.1 mL to χ^2 = 5.8 when comparing weep with rinse with 0.5 mL.

The weep sample enumeration distribution of *Campylobacter*, by exponential groupings, among the processed carcasses is shown in Figure 3. The data represent the distribution for exponential levels of contamination. The actual estimations for levels of *Campylobacter* per carcass for each process lot, comparing weep and rinse methods, are shown in Table 2. Process lot number 25 had the highest levels observed with an average of $10^{4.51}$ *Campylobacter* cfu/mL of weep and $10^{5.94}$ cfu in 100-mL rinses.

Genetic typing of isolates revealed little difference between sampling by the weep and rinse methods. For 1

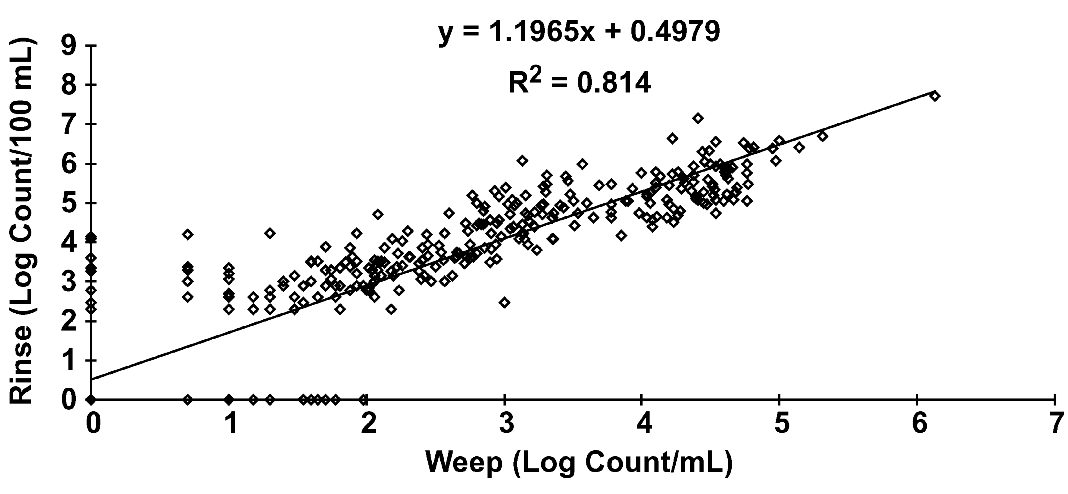

Figure 2. Regression analysis of rinse and weep log counts from 374 broiler carcasses contaminated with *Campylobacter* spp. as measured per milliliter of weep fluids or by enumerating 0.5 of 100 mL of rinse fluids inoculated onto Campy-Cefex agar.

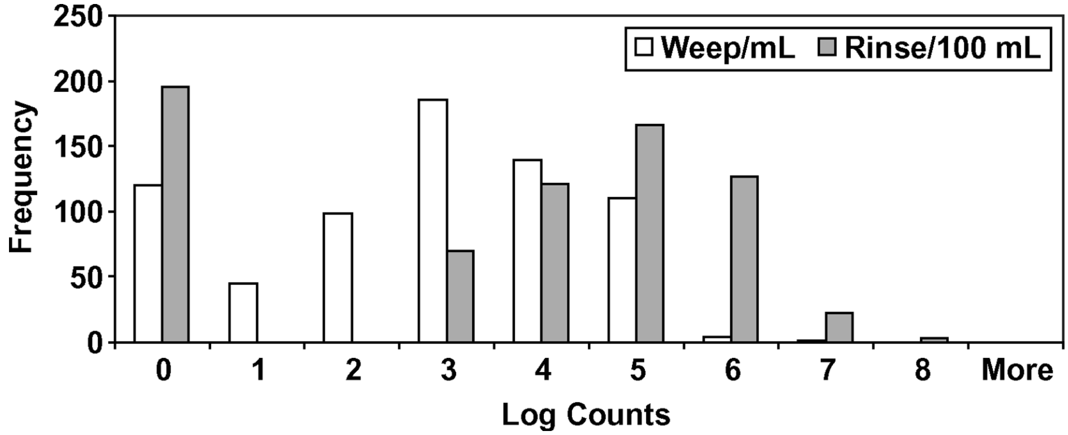

Figure 3. Enumeration distribution of *Campylobacter* by exponential groupings, among known contaminated carcasses as measured by enumeration of free weep and rinse samples from fresh, fully processed, Iceland broiler chickens.

flock, all 23 weep isolates, 12 rinse isolates, and 4 cecal isolates had the same *flaA* SVR allele (A11203). In the second flock, 16 of the 17 weep isolates, 4 of the 5 rinse isolates, and all 4 of the cecal isolates had 1 allele (A15498), whereas the remaining weep isolate has an allele (A14898) that differed from the common allele by 1 substitution. The last rinse isolate had an allele (A14875) that differed from A15498 by 74 substitutions. Because we have only 4 preprocessing isolates, we cannot know whether alleles A14898 and A14875 were present in the flock prior to slaughter or if they represented cross-contamination during processing. It did appear that the rinse and weep methods of sampling retail product could identify the predominant allele without difficulty and that both could provide common alleles present on the carcass.

DISCUSSION

What monitoring measure should food inspection services use? And what is an acceptable level of risk for *Campylobacter* carcass contamination? These questions disregard the established understanding that there will, inevitably, be differences in human-virulence expression among specific strains. There will also be differences in human susceptibility to specific strains. Such answers will require additional insight and resource investment to resolve. However, consumers will equally mishandle poultry products, regardless of the unknown levels of pathogen contamination on the product. Weep and rinse sample enumerations (Figure 2) were well correlated, and sequence typing of isolates recovered using the 2 methods also gave similar results. The results of studies conducted using one of the sampling regimens would be acceptable to monitor carcass contamination.

Health concerns over the organism are expressed in a recent World Health Organization (2005) surveillance program newsletter article which indicated that qualitative and quantitative occurrence of *Campylobacter* in broiler chickens along the farm-to-fork continuum should be collected and validated. Pursuant to the question of *Campylobacter* numbers, Nannapaneni et al. (2005) reported that total colony-forming units of ciprofloxacin-resistant *Campylobacter* on carcass rinses in Arkansas ranged up to 4.06, 3.95, and to 3.04 $\log_{10}$ cfu/carcass in 2001, 2002, and 2003, respectively. Jorgensen et al. (2002) reported levels of *Campylobacter* on carcasses from retail outlets in England during the winters of 1998 and 1999. In that study, the $\log_{10}$ colony-forming units of *Campylobacter* spp. were 2.70 to 4.99 in 18% of the chickens and 5.00 to 6.99 in 20% of the carcasses sampled. In Italy, among 213 carcasses sampled, the overall estimated mean count per carcass was 5.16 ± 0.80, with the greatest level per flock averaging 6.13 $\log_{10}$ cfu/carcass (Manfreda et al., 2006). Stern and Robach (2003) reported the average numbers of *Campylobacter* spp. per broiler carcass in northern Georgia had decreased from 4.11 to 3.05 $\log_{10}$ cfu from 1995 to 2001. Throughout the United States from September 2003 through September 2004, 4,200 carcasses were sampled, and approximately 3.6% of the commercially processed broiler carcasses had greater than 10^5 cfu of *Campylobacter* spp. per carcass (Stern and Pretanik, 2006). In the present

Table 1. Comparison of qualitative recoveries of *Campylobacter jejuni* from processed carcasses by using weep and 0.1 or 0.5 mL-rinse sampling procedures[1]

	Weep isolation/carcass		
Rinse isolation/carcass	+	−	Total
0.1 mL of inoculum[2]			
+	232	2	234
−	63	33	96
Total	295	35	330
0.5 mL of inoculum[3]			
+	265	10	275
−	24	75	99
Total	289	85	374

[1]Direct plating of weep and rinse samples onto Campy-Cefex agar was used for detection.

[2]Weep isolation = 89.4% (295/330) with a 95% confidence interval (CI) of [86.1%, 92.7%]; rinse isolation = 70.1% (234/330) with a 95% CI of [66.0%, 75.8%]; McNemar test χ^2 = 57.246 (P = 0.0000).

[3]Weep isolation = 77.3% (289/374) with a 95% CI of [73.0%, 81.5%]; rinse isolation = 73.5% (275/374) with a 95% CI of [69.1%, 78.0%]; McNemar test = 5.765 (P = 0.0164).

Table 2. Enumeration of *Campylobacter* spp. associated with process lots of fresh, fully processed broiler carcasses as estimated by weep and rinse methods

Batch ID	Date	Weep[1] (average log/mL)	n	Rinse (average log/100 mL)	n
1	July 9, 2003	1.39 ± 0.65	19	3.28 ± 0.56	4
2	July 18, 2003	4.17 ± 0.55	20	5.05 ± 0.48	20
3	July 21, 2003	3.41 ± 0.46	20	4.34 ± 0.39	20
4	July 25, 2003	2.23 ± 0.80	19	3.56 ± 0.82	12
5	July 25, 2003	2.37 ± 0.79	19	3.49 ± 0.87	10
6	July 30, 2003	2.86 ± 0.56	20	4.06 ± 0.55	17
7	September 1, 2003	3.24 ± 0.72	18	4.74 ± 0.80	20
8	September 2, 2003	3.04 ± 0.69	19	4.96 ± 1.01	18
9	September 4, 2003	2.42 ± 0.65	18	3.63 ± 0.59	14
10	September 19, 2003	2.51 ± 0.78	8	3.89 ± 0.81	9
11	September 25, 2003	2.92 ± 0.67	19	4.49 ± 0.58	20
12	October 15, 2003	4.11 ± 0.38	20	5.55 ± 0.66	20
13	October 17, 2003	1.09 ± 0.37	15	3.21 ± 0.24	6
14	October 24, 2003	3.29 ± 0.58	19	4.90 ± 0.69	20
15	January 9, 2004	1.84 ± 0.72	14	3.62 ± 0.59	7
16	February 22, 2004	2.02 ± 0.51	8	4.10 ± 0.75	7
17	May 7, 2004	3.17 ± 0.49	20	4.62 ± 0.80	20
18	July 14, 2004	4.33 ± 0.35	20	5.24 ± 0.52	20
19	July 16, 2004	1.82 ± 0.59	20	3.28 ± 0.58	20
20	July 22, 2004	2.11 ± 0.64	20	3.47 ± 0.75	16
21	July 23, 2004	3.72 ± 0.88	20	5.37 ± 0.91	20
22	August 6, 2004	2.23 ± 0.60	20	3.19 ± 0.83	19
23	August 6, 2004	2.15 ± 0.81	18	3.79 ± 0.56	20
24	August 11, 2004	3.66 ± 0.73	20	4.76 ± 0.79	20
25	August 12, 2004	4.51 ± 0.37	20	5.94 ± 0.53	20
26	August 13, 2004	3.08 ± 0.49	20	4.17 ± 0.71	20
27	August 20, 2004	3.64 ± 0.43	19	4.88 ± 0.53	20
28	August 25, 2004	1.62 ± 0.50	17	2.95 ± 0.49	12
29	August 26, 2004	3.10 ± 0.41	20	4.98 ± 0.59	20
30	September 9, 2004	4.50 ± 0.19	16	5.27 ± 0.34	20
31	September 10, 2004	1.81 ± 0.63	14	3.47 ± 0.74	17
32	September 29, 2004	1.52 ± 0.54	18	3.06 ± 0.46	17

[1]Only log counts from positive specimens are included in these averages.

study among known contaminated carcasses, the average rinse numbers per carcass lot ranged from 2.95 to 5.94 $\log_{10}$ cfu (Table 2) with 41, 38, and 19% of the lots with results between 3 to 4, 4 to 5, and 5 to 6 $\log_{10}$, respectively.

Results from a previous study in Iceland by Georgsson et al. (2006) showed among 5 processed broiler lots (50 carcasses), the average carcass rinse per lot ranged from 4.66 to 6.07 $\log_{10}$ and was distributed as 50, 32, 14, and 4% among 4 to 5, 5 to 6, 6 to 7, and 7 to 8 $\log_{10}$, respectively. These earlier results for fresh broilers in Iceland were considerably higher than found in the present study. One explanation for the reduced numbers seen in the present study is that the flocks tested were *Campylobacter* negative at ~32 d of age and only became colonized during the last 4.1 ± 0.9 d before slaughter, whereas flocks in the previous study were demonstrated as positive prior to ~32 d of age. Colonization levels would be expected to increase with additional rearing time (Stern et al., 2005). An additional conclusion posed is that even though a control program to exclude naturally contaminated broilers from the market (by testing production flocks at ~32 d) cannot assure all fresh broilers are *Campylobacter* free, our data indicate that such inspection and altering how the product is distributed will reduce the level of public exposure.

The World Health Organization has suggested that weep fluids from processed poultry may provide a superior estimate of consumer exposure to *Campylobacter* spp. Regulatory Agencies in both Iceland and the United States routinely use rinse methodology to assess the microbiological quality of poultry carcasses in the processing plants. Weep sampling requires packaging and storing the carcasses until the weep fluid accumulates in the packaging material (~24 h). The package must then be aseptically opened and the weep aspirated for microbiological sampling. The quantity of weep materials produced is highly variable among each carcass and is, in part, dependent upon how many abrasions the carcass received during its processing, how well the carcass was drained after chilling, and the length of time the carcass was held in storage.

Weep samples may vary from 0.5 mL to several milliliters per carcass. As a confounder, the *Campylobacter* enumeration may vary with the amount of weep accumulated. To accomplish their goals, inspectors among regulatory agencies must work in a timely manner and must have a consistent sampling protocol. The variation in weep sample quantity disregards this requirement. Conversely, rinse samples are typically taken at the end of the broiler processing line and consistent quantities of diluent are added to individual carcasses. The carcass rinse agitation is prescribed for a certain arc and duration. This rinse sampling remains the standard and, as shown in this paper, has substantial agreement with the weep sample method. When 0.5 mL of direct plating was used for quantitative rinse analyses, the substantial regression with the weep plating ($R^2 = 0.814$) further supported the traditional sampling approach. When combined with the correlation coefficients indicated, this regression analysis enabled us to conclude that the 2 sample methods were highly predictive of one another. Despite the fact weep and rinse sampling approaches led to different results in terms of the proportion of *Campylobacter*-positive carcasses, it was demonstrated that this difference was reduced by increasing the amount of fluid used for the rinse method.

Regulatory agencies and the poultry industries are increasingly concerned over what levels of *Campylobacter* associated with broiler carcasses is a human health hazard. Answers to this question should be made using an acceptable method that can be incorporated into an ongoing regulatory regimen. After a method has been accepted, a rigorous comparison of poultry-borne *Campylobacter* exposure levels to the risk of human disease will be needed to draw the line between limited risk and substantial risk related to contamination of poultry carcasses.

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